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Analytical Method

TEST GUIDELINE(S): OECD ENV/JM/MONO(2007)17
EPA 850.6100
EC SANCO/3029/99 rev 4
EC SANCO/825/00 rev 8.1

1.0 INTRODUCTION

1.1 Scope of the Method

Analytical method GRM072.17A is suitable for the determination of SYN547407 and Metabolites SYN549107, SYN549431, SYN550455 and SYN551485 (Figure 1-5) in water. The limit of quantification (LOQ) of the method has been established at 0.05 μg/L (or 0.05 ppb).

This method satisfies US EPA guideline EPA OCSPP 850.6100 and EC Guidance Documents SANCO/3029/99 rev 4 and SANCO/825/00 rev 8.1.

Method includes in-house generated validation data for surface water and ground (drinking) water.

1.2 Stereospecificity

This method does not resolve the diastereomers or the R and S enantiomers of each diastereomeric pair and will detect and quantify all enantiomers of each compound as a single chromatographic peak with the exception of SYN550455 where baseline separation occurs under reverse phase conditions.

1.3 Method Summary

Water samples are direct diluted with acetonitrile then submitted for final determination by high performance liquid chromatography with triple quadrupole mass spectrometric detection (LC-MS/MS).

The limit of quantification of the method is 0.05 μg/L (0.05 ppb).

2.0 MATERIALS AND APPARATUS

2.1 Apparatus

The recommended equipment and apparatus are listed in Appendix 1. Equipment with equivalent performance specifications may be substituted.

2.2 Reagents

All solvents and other reagents must be of high purity, e.g. glass distilled/HPLC grade solvents and analytical grade reagents. Particular care must be taken to avoid contamination of the reagents used. Reagents of comparable purity may be substituted as long as acceptable performance is demonstrated. A list of reagents used in this method along with details of preparation of solutions is included in Appendix 2.

2.3 Safety Precautions and Hazards

The following information is included as an indication to the analyst of the nature and hazards of the reagents used in this procedure. If in any doubt, consult the appropriate SDS.

2.3.1 Solvent and Reagent Hazards

The following information is included as an indication of the hazards associated with the reagents used in this procedure. The procedure is intended to be used by trained and competent personnel, well versed in laboratory safety. Users of the method should ensure they have sufficient information to conduct an appropriate risk assessment that fulfils local requirements. If in any doubt, consult a suitably qualified expert for further advice

It is recommended that the following precautions should be taken when handling the analytical standards and reagents.

- Avoid breathing dust of vapors; ensure good ventilation at all times.
- Avoid skin contact; wear suitable gloves when handling.
- Prevent contamination of skin and clothing: wear a laboratory coat.
- Avoid contact with mouth; ensure good laboratory hygiene practice.
- Avoid spillages; wash any contaminated area immediately with tissue soaked with an appropriate solvent.

The following hazards have been identified for the solvents and reagents utilised in this method.

Compound	**Hazard Phrases**
Acetonitrile	H225: Highly flammable liquid and vapour H302: Harmful if swallowed H312: Harmful in contact with skin H316: Causes mild skin irritation H318: Causes serious eye damage H332: Harmful if inhaled
Acetic Acid	H226: Flammable liquid and vapour H314: Causes severe skin burns and eye damage H318: Causes serious eye damage H402: Harmful to aquatic life

2.3.2 Analytical Standard Material Hazards

Hazard information is not available for SYN547407 and metabolites. It is recommended that all analytical standard materials are treated as hazardous and appropriate control measures are used to reduce the risk of exposure. The following minimum precautions should be taken when handling the analytical materials directly.

- Avoid breathing dust or vapors; ensure good ventilation at all times.
- Avoid skin contact; wear suitable gloves when handling.
- Prevent contamination of skin and clothing: wear a laboratory coat.
- Avoid contact with mouth; ensure good laboratory hygiene practice.
- Avoid spillages; wash any contaminated area immediately with tissue soaked with an appropriate solvent.

2.4 Preparation of Analytical Standard Solutions

2.4.1 Stock Solutions

Prepare a 100 μg/mL stock solution for each compound, by one of the following methods.

Note: the amount weighed out must be corrected for the purity of the analytical standard as indicated on the certificate of analysis and also any salt content, where the analytical standard is received as a salt e.g. Na^+, Cl^- etc.

Weigh out accurately, using a five-figure balance, sufficient standard material into separate amber "Class A" volumetric flasks (50 mL size). Dilute to the mark with acetonitrile to give a 100 μg/mL stock solution of each individual compound.

Alternatively, the appropriate volume of acetonitrile to add to a known amount of standard material may be determined using the equation below. The standard concentration is corrected for its chemical purity and any salt content where the analytical standard is received as a salt e.g. Na^+, Cl^- etc.

$$V = \frac{W \times P}{C} \times 1000$$

P	=	Standard purity (including correction for salt content where the analytical standard is received as a salt e.g. Na^+, Cl^- etc.) in decimal form (P(%)/100)
V	=	Volume of acetonitrile required
W	=	Weight, in mg, of the solid analytical standard
C	=	Desired concentration of the final solution, (μg/mL)
1000	=	Unit conversion factor

In this case, the standard material is weighed directly into an appropriate storage vessel.

2.4.2 Fortification Solutions

Combined fortification solutions containing SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 should be prepared by serial dilution in acetonitrile. It is recommended that the following solutions are prepared: 0.10 μg/mL, 0.010 μg/mL and 0.001 μg/mL.

2.4.3 Preparation of Calibration Standards for LC-MS/MS

No significant suppression or enhancement of the instrument response has been observed in the water types tested using the procedures described in Section 3 during method validation and solvent based standards should normally be used for calibration.

Solvent based calibration standards should be prepared by serial dilution of stock or fortification solutions to a final solution of acetonitrile/ultra-pure water (50/50 v/v).

For example, to prepare a standard solution at 1.0 µg/L, transfer an aliquot (1.0 mL) of the 0.01 µg/mL fortification standard into a 10 mL volumetric flask, add 1 mL of ultra-pure water and dilute to 10 mL mark using acetonitrile/ultra-pure water (50/50 v/v).

The recommended calibration range for analysis using the instrumentation described in Section 4 for matrix addition is: 0.005 µg/L to 5.0 µg/L (0.15 pg to 150 pg on column based on a 30 µL injection volume).

A calibration curve should be generated to quantify residues. Standards over an appropriate concentration range in acetonitrile/ultra-pure water (50/50 v/v) should be prepared as described above.

Any matrix effects observed may be compensated for by use of matrix-matched standards at the discretion of the study director, or by dilution of the final sample with acetonitrile/ultra-pure water (50/50 v/v) should instrument sensitivity permit.

2.4.4 Standard Solution Storage and Expiration

All stock solutions should be stored in a refrigerator or freezer when not in use to prevent decomposition and/or concentration of the standard. Standard solutions should be allowed to equilibrate to room temperature prior to use.

An expiration date of 3 months for SYN547407 and metabolites in acetonitrile/ultra-pure water (50/50 v/v) is recommended (Reference 1) unless additional data are generated to support a longer expiration date.

3.0 ANALYTICAL PROCEDURE

A summary of the method is included in flow-chart form in Appendix 4.

3.1 Sample Collection

a) Due to possible adsorption of compound(s) to plastic (HDPE and PP) it is highly recommended that water samples be collected and stored in glass.

3.2 Sample Preparation

a) If water samples are received deep frozen, they should be allowed to defrost completely at room temperature. Defrosted samples should be shaken thoroughly to ensure sample homogeneity prior to analysis.

3.3 Sample Fortification and Dilution

a) Transfer 0.5 mL of the water sample to an autosampler vial. Sample fortification, if required, is to be carried out at this time using 0.1 mL of fortification solution in acetonitrile.

b) Adjust final volume to 1.0 mL using acetonitrile.

c) Final sample solution is acetonitrile/ultra-pure water (50:50 v/v).

d) Transfer an aliquot into a suitable autosampler vial for final determination by LC-MS/MS.

3.4 Experimental Precautions

a) To prevent contamination of the instrument and to minimize possible carry-over issues, it is recommended that high level recoveries (>5 µg/L) and samples with expected residues greater than 5 µg/L should be diluted so that the final analyte concentration does not exceed 5 µg/L. It may also be useful to include blank injections of acetonitrile/ultra-pure water (50/50 v/v) after high level samples to clear any observed carry-over greater than 10% of the LOQ.

3.5 Time Required for Analysis

The methodology is normally performed with a batch of 30 samples. One person can complete the analysis of 30 samples in 1 day (8 hour working period).

3.6 Method Stopping Points

The analytical procedure can be stopped at various points for overnight and weekend breaks unless otherwise specified in the analytical procedure. Acceptable method recoveries will validate any workflow interruptions. Samples should be stored refrigerated in sealed containers where the analysis cannot be completed in a single day.

4.0 FINAL DETERMINATION

The method has been developed for use on an Applied Biosystems QTRAP® 6500 using the direct dilution procedure. The following instrumentation and conditions have been found to be suitable for this analysis. Other instrumentation can also be used, though optimization may be required to achieve the desired separation and sensitivity. The operating manuals for the instruments should always be consulted to ensure safe and optimum use.

4.1 Instrument Description

LC System	:	Sciex ExionLC™
Detector	:	Applied Biosystems QTRAP® 6500
Gas Supply	:	Nitrogen/Air Generator

4.2 Chromatography Conditions for SYN547407

Column	:	Phenomenex Kinetex C18 50 x 4.6 mm 2.6 µm
Column Oven Temperature	:	30 °C
Injection volume	:	30 µL
Stop Time	:	7 minutes
Injection protocol	:	Analyze calibration standard after 3 to 4 sample injections*
Mobile phase	:	(A) Ultra-Pure Water + 0.01% Acetic Acid (B) Acetonitrile + 0.01% Acetic Acid

Mobile Phase Composition

Time (mins)	% A	% B	Flow rate (mL/min)
0	90	10	1.0
0.5	90	10	1.0
3.0	30	70	1.0
5.0	5	95	1.0
6.0	5	95	1.0
6.1	90	10	1.0
7.0	90	10	1.0

If polarity switching does not provide enough data points across the target peak(s), separate injections in negative mode and positive mode may be required.

Compound Retention Time

Analyte	Minutes
SYN550455	3.4
SYN549431	3.9
SYN547407	4.1
SYN551485	4.1
SYN549107	4.3

4.3 Mass Spectrometer Conditions

Interface	:	TurboIonSpray
Curtain gas (CUR)	:	Nitrogen set at 20 (arbitrary units)
Temperature (TEM)	:	600°C
Ionspray voltage	:	5500V Positive Mode /-4500V Negative Mode
Collision gas setting (CAD)	:	Nitrogen set at Medium (arbitrary units)
Gas 1 (GS1)	:	Air set at 60 (arbitrary units)
Gas 2 (GS2)	:	Air set at 50 (arbitrary units)
Interface heater (ihe)	:	On
Scan type	:	MRM

MRM Conditions		**SYN547407 primary transition**	**SYN547407 confirmatory transition**	**SYN549107 primary transition**	**SYN549107 confirmatory transition**
Q1 *m/z*	:	548	548	434	434
Q3 *m/z*	:	418	160	354	238
Dwell time	:	25 ms	25 ms	25 ms	25 ms
Resolution Q1	:	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	40 V	40 V	-91 V	-91 V
Entrance potential (EP)	:	10 V	10 V	-10 V	-10 V
Collision energy (CE)	:	37 V	55 V	-30 V	-30 V
Collision cell exit potential (CXP)	:	14 V	10 V	-10 V	-10 V

MRM Conditions		**SYN549431 primary transition**	**SYN549431 confirmatory transition**	**SYN550455 primary transition**	**SYN550455 confirmatory transition**
Q1 *m/z*	:	435	435	553	553
Q3 *m/z*	:	392	160	277	247
Dwell time	:	25 ms	25 ms	25 ms	25 ms
Resolution Q1	:	Unit	Unit	Unit	Unit
Resolution Q3	:	Unit	Unit	Unit	Unit
Declustering potential (DP)	:	60 V	60 V	-160 V	-160 V
Entrance potential (EP)	:	14 V	14 V	-10 V	-10 V
Collision energy (CE)	:	33 V	45 V	-25 V	-40 V
Collision cell exit potential (CXP)	:	10 V	10 V	-10 V	-11 V

MRM Conditions		**SYN551485 primary transition**	**SYN551485 confirmatory transition**
Q1 *m/z*	:	503	503
Q3 *m/z*	:	390	431
Dwell time	:	25 ms	25 ms
Resolution Q1	:	Unit	Unit
Resolution Q3	:	Unit	Unit
Declustering potential (DP)	:	-60	-60
Entrance potential (EP)	:	-10	-10
Collision energy (CE)	:	-25	-20
Collision cell exit potential (CXP)	:	-10	-10

Typical chromatograms are shown in the Figures Section.

4.4 Confirmatory Procedures

Final determination by LC-MS/MS with two transitions is considered to be highly specific; hence no further confirmatory conditions are included.

5.0 CALCULATION OF RESULTS

5.1 Multi-Point Calibration Procedure

Compound residues may be calculated in µg/L for each sample as follows.

a) Prepare standard solutions over a concentration range appropriate to the expected residues in the samples (30% LOQ to at least 20% above the highest fortified level as a minimum). An appropriate number of different concentrations within this range should be prepared (at least five).

b) Make an injection of each sample solution and measure the areas of the peaks corresponding to each compound. Calibration standard solutions should be interspersed throughout the analysis, after a maximum of four injections of sample solutions.

c) Generate calibration curve parameters using an appropriate regression package.

d) The following equation can be rearranged and used to calculate residues as follows:

$$y = mx + c$$

Where y is the instrument response value, x is the standard concentration, m is the gradient of the line of best fit ("X-variable 1" in MS Excel) and c is the intercept value. An example of this equation generated using the experimental values of m and c should be included in the raw data, as should the "R-Squared" value for the regression.

Re-arrangement for *x* gives

$$x = \frac{y - c}{m}$$

e) Calculate the compound specific residues in the sample, expressed as µg/L, as follows

$$\text{Residue } (\mu\text{g/L}) = \frac{\text{Analyte found } (\mu\text{g/mL})}{\text{Sample conc. (L/mL)}}$$

Where analyte found (µg/mL) is calculated from the standard calibration curve and sample conc. is the final sample concentration in L/mL.

If residues need to be corrected for average percentage recovery e.g. for storage stability studies, then the equation below should be used.

$$\text{Corrected Residue} = \frac{\text{Residue} \times 100}{\text{Average percentage Recovery}} \ (\mu\text{g/L})$$

6.0 CONTROL AND RECOVERY SAMPLES

Control samples should be analyzed with each set of samples to verify that the sample used to prepare recovery samples is free from contamination. A minimum of one control should be analyzed with each batch of samples.

At least two recovery samples (control samples accurately fortified with known amounts of SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 in acetonitrile) should also be analyzed alongside each set of samples to verify the performance of the method. Provided the recovery values are acceptable they may be used to correct any residues found. The fortification levels should be appropriate to the residue levels expected.

Recovery efficiency is generally considered acceptable when the mean values are between 70% and 120% and with a relative standard deviation of $\leq$20%.

Where the method is used for monitoring purposes, control and recovery samples are not required where suitable control samples are not available.

7.0 SPECIFICITY

It is recommended that reagent blank samples be included in a sample set if contamination is suspected.

7.1 Matrix

LC-MS/MS is a highly specific detection technique. Interference arising from the matrices tested has not been observed.

7.2 Reagent and Solvent Interference

Using high purity solvents and reagents no interference has been found.

7.3 Labware Interference

All reusable glassware should be detergent washed and then rinsed with HPLC grade methanol, acetone or acetonitrile prior to use.

8.0 METHOD VALIDATION

8.1 Extractability

Residues of SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 in water are analyzed by LC-MS/MS following dilution with acetonitrile and extractability is not relevant.

Acceptable recovery data from the method validation verify the performance of the method for determination of SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 in water.

8.3 Limit of Quantification (LOQ)

The limit of quantification of the method is defined as the lowest analyte concentration in a sample at which the methodology has been validated and a mean recovery of 70-120% with a relative standard deviation of ≤ 20% has been obtained.

The limit of quantification has been set at 0.05 μg/L (0.05 ppb).

8.4 Limit of Detection (LOD)

The limit of detection of the method is defined as the lowest analyte concentration detectable above the mean amplitude of the background noise in an untreated sample at the corresponding retention time. An estimate of the LOD can be taken as three times background noise. Note that the LOD may vary between runs and from instrument to instrument.

The LOD was determined as 0.15 pg injected on column, equivalent to 0.005 μg/mL when using a 30 μL injection volume. LOD is based on the lowest concentration sample injected.

8.5 Matrix Effects

No significant matrix effects were observed in the water types tested during method validation and non-matrix standards should generally be used for quantification. A summary of the matrix effects is included in Table 6.

The method includes procedures to reduce matrix effects as far as practically possible, however matrix effects in some matrices may be still observed. In these instances, matrix matched standards may be used to compensate for the matrix effects at the discretion of the study director.

8.6 Detector Linearity

For accurate quantification of residue concentrations, analyses should be carried out within the linear range of the detector. For multi-point calibration, detector range and linearity will be demonstrated within each sample set.

The linearity of the LC-MS/MS detector response for all compounds were tested in the range from 0.15 pg to 150 pg injected on column (equivalent to 0.005 μg/L to 5 μg/L standards when using a 30 μL injection volume) and was found to be linear. This is equivalent to 20% LOQ to 100X LOQ (i.e. at least 30% LOQ - 20% above the highest validated level).

If a residue beyond the tested concentration range is expected, dilute the sample appropriately to bring it within the tested linear range prior to quantification.

Inject standards in the range of 30% LOQ – 20% above highest concentration required and plot the peak area against the standard concentration, using Microsoft Excel for both primary and confirmatory transitions for SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485.

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8.7 Extract Stability

Final water samples in acetonitrile/ultra-pure water (50/50 v/v) retained in vials and stored at a temperature of approximately 4ºC were suitable for SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 residue analysis, for storage periods of up to 7 days. A summary of the stability data obtained during method validation is presented in Table 7 and 8.

9.0 LIMITATIONS

The method has been tested on representative water types. It can reasonably be assumed that the method can be applied to other water matrices not tested in this method provided successful recovery tests at the relevant levels validate the suitability of the method.

10.0 CONCLUSIONS

This procedure has been demonstrated to be a reliable and accurate procedure for the determination of SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 residue in water. Only commercially available laboratory equipment and reagents are required.

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satisfies US EPA guideline EPA OCSPP 850.6100 and EC Guidance Documents SANCO/3029/99 rev 4 and SANCO/825/00 rev 8.1.

TABLE 1: Characterization Data of Water Samples used for Method Validation.

Matrix	Source	pH	Calcium	Magnesium	Sodium	Hardness	Conductivity	SAR
Ground water	Residential	7.3	9.2 ppm	2.0 ppm	6.8 ppm	31 mg	0.10 mmhos/cm	0.53
Surface water	Local Lake	7.5	7.4 ppm	3.5 ppm	6.7 ppm	33 mg	0.13 mmhos/cm	0.51

Determination of LC-MS/MS Matrix Effects

The effect of different water types on the LC-MS/MS signal was assessed by preparing standards in the presence of water matrix and comparing the peak areas of SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 against non-matrix standards at an equivalent concentration.

Note: % matrix effects determined as (B/A x 100) – 100 where A is non-matrix standard response and B is matrix-matched standard response

TABLE 6: LC-MS/MS Matrix Effects

	Matrix Effect	
Analyte	Ground water	Surface Water
SYN547407 *m/z* 548 → 418	+8	+12
SYN547407 *m/z* 548 → 160	-10	+1
SYN549107 *m/z* 434 → 354	+7	+4
SYN549107 *m/z* 434 → 238	+4	+5
SYN549431 *m/z* 435 → 392	+6	-2
SYN549431 *m/z* 435 → 160	+4	+2
SYN550455 *m/z* 553 → 277	+1	-1
SYN550455 *m/z* 553 → 247	+4	+1
SYN551485 *m/z* 503 → 390	+3	+5
SYN551485 *m/z* 503 → 431	+4	+4

The magnitude of the matrix effects was considered not to be significant for the water types tested and non-matrix standards were used for calibration during method validation. Matrix matched standards may be used to compensate for any significant effects, at the discretion of the study director. Alternatively, where instrument sensitivity permits, samples may be further diluted in acetonitrile/ultra-pure water (50/50 v/v) to reduce or eliminate these effects.

CHEMICAL STRUCTURES

FIGURE 1: SYN547407

Alternative compound code number	**:**	CSCV765754
CAS Number	**:**	Not in registry
IUPAC Name	**:**	4-(5-(3,5-dichloro-4-fluorophenyl)-5-(trifluoromethyl)-4,5-dihydro-1,2-oxazol-3-yl)-N-(2-ethyl-3-oxo-1,2-oxazolidin-4-yl)-2-methylbenzamide
Molecular Formula	:	$C_{23}H_{19}O_4N_3Cl_2F_4$
Molecular Weight	**:**	548.32

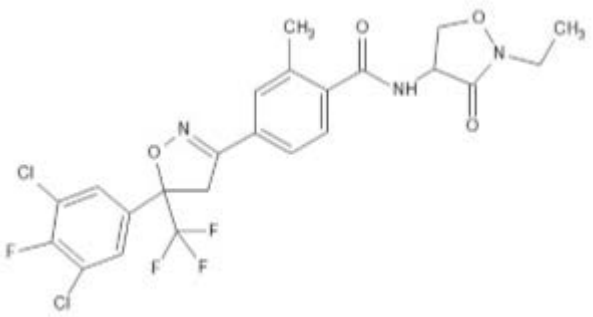


FIGURE 2: SYN549107

Alternative compound code number	**:**	CSCD747695
CAS Number	**:**	Not in registry
IUPAC Name	**:**	4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4H-isoxazol-3-yl]-2-methyl-benzoic acid
Molecular Formula	:	$C_{18}H_{11}O_3NCl_2F_4$
Molecular Weight	**:**	436.18

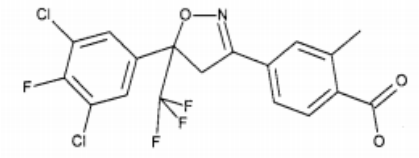


FIGURE 3: SYN549431

Alternative compound code number	**:**	CSDK352552
CAS Number	**:**	Not in registry
IUPAC Name	**:**	4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4H-isoxazol-3-yl]-2-methyl-benzamide
Molecular Formula	:	$C_{18}H_{12}O_2N_2Cl_2F_4$
Molecular Weight	**:**	435.20

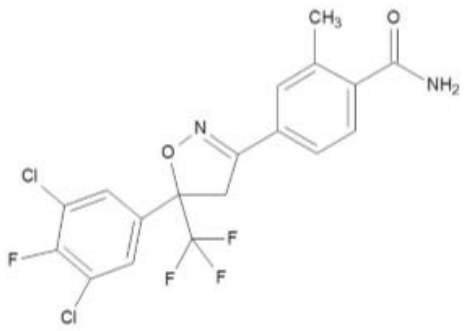


FIGURE 4: SYN550455

Alternative compound code number	**:**	CSDK388837
CAS Number	**:**	Not in registry
IUPAC Name	**:**	4-[3-(3,5-dichloro-4-fluoro-phenyl)-4,4,4-trifluoro-1,3-dihydroxy-butyl]-N-[2-(ethylamino)-1-(hydroxymethyl)-2-oxo-ethyl]-2-methyl-benzamide
Molecular Formula	:	$C_{23}H_{24}O_5N_2Cl_2F_4$
Molecular Weight	**:**	555.35

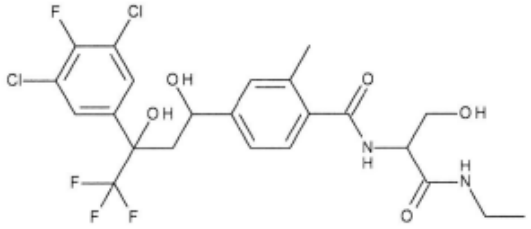
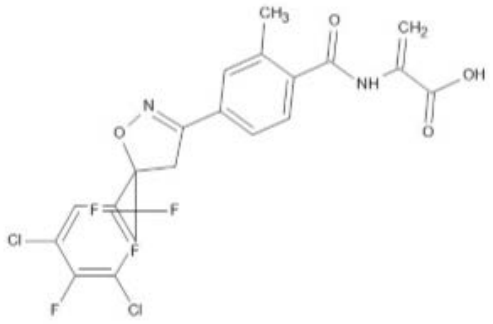


FIGURE 5: SYN551485

Alternative compound code number	**:**	CSCV765754
CAS Number	**:**	Not in registry
IUPAC Name	**:**	2-[[4-[5-(3,5-dichloro-4-fluoro-phenyl)-5-(trifluoromethyl)-4H-isoxazol-3-yl]-2-methyl-benzoyl]amino]prop-2-enoic acid
Molecular Formula	:	$C_{21}H_{14}O_4N_2Cl_2F_4$
Molecular Weight	**:**	505.25



APPENDIX 1 APPARATUS

Recommended Suppliers

Equipment	Description	Supplier
General glassware	General glassware	Thermo Fisher
Autosampler Vials	1.5-mL vials/caps	Thermo Fisher
LC-MS/MS system	AB Sciex Q Trap® 6500+	Sciex
HPLC system	ExionLC	Sciex
LC Column	Kinetex C18 column (50 mm x 4.6 mm i.d, 2.6 µm particle size). Part number 00B-4462-EO.	Phenomenex
Nitrogen/Air	House	Syngenta

APPENDIX 2 REAGENTS

Recommended Suppliers

Reagent	Description	Supplier
Ultra-pure water	Optima/HPLC grade	Thermo Fisher
Acetonitrile	Optima/HPLC grade	Thermo Fisher
Acetic acid	Optima/HPLC grade	Thermo Fisher
Analytical standard	GLP certified	Syngenta Crop Protection, Inc., P.O. Box 18300, Greensboro, NC 27419-8300.

Preparation of Reagents

a) Acetonitrile/ultra-pure water (50/50 v/v) – prepared by mixing 500 mL of purified water with 500 mL of acetonitrile. (Dilution Solvent)

b) 0.01% acetic acid in ultra-pure water – add 0.10 mL concentrated formic acid to 1L ultra-pure water. (Mobile Phase)

c) 0.01% acetic acid in acetonitrile – add 0.10 mL concentrated formic acid to 1L ultra-pure water. (Mobile Phase)

APPENDIX 3 LC-MS/MS TUNING PROCEDURE

Calibration of Instrument

The instrument must be mass calibrated on a regular basis using polypropylene glycol (PPG) solutions according to the manufacturer's instructions. Calibrate both mass resolving quadrupoles (Q1 and Q3).

Tuning Instrument for SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485

Note: SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 have 2 chlorine atoms. Chlorine has two isotopic forms ^{35}Cl and ^{37}Cl On the instruments tested, the most abundant ion is for M (i.e.^{35}Cl x 2).

Infuse standard solutions of SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 (0.01 to 1.0 µg/mL) in mobile phase (see section 4) directly into the mass spectrometer interface at a rate at of approximately 10-20 µL/min. Roughly adjust interface parameters (sprayer position, spray, heater/auxiliary gas flows, as well as voltages of spray, orifice, and focusing ring) for a sufficiently high molecular ion signal for each compound.

Finally, connect the LC-pump via the autosampler directly to the MS/MS instrument. Perform repetitive flow injection SYN547407, SYN549107, SYN549431, SYN550455 and SYN551485 standards using mobile phase at the flow rate to be used. Tune the interface parameters (sprayer position, spray and heater gas flows, spray, orifice, and focusing ring voltages) and the collision gas flow for maximum sensitivity. In negative ionization mode, the deprotonated molecular ion generated in the ion source for each compound is selected and subjected to further fragmentation by collisional activation. The two most sensitive product ions are then selected and used for quantitative analysis.

Metabolite	Molecular Ion	Product Ion	Confirmatory Ion
SYN547407	548	418	160
SYN549107	434	354	238
SYN549431	435	392	160
SYN550455	553	277	247
SYN551485	503	390	431